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Benzo(a)Pyrene Metabolism in Marine Fish and Some Analytical Aspects of Its Metabolites

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Benzo[a]pyrene Metabolism in Marine Fish and Some Analytical Aspects of Its Metabolites[1, 2]

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ABSTRACT—In interspecies comparisons of benzo[a]pyrene metabolism, standardized procedures in the analysis of the metabolites must be established. Technical problems arising in metabolite analysis are discussed, and data are presented showing the activities of the mixed function oxygenase and transferase systems in phenobarbital- and 3-methylcholanthrene-induced mullet and killifish. — Natl Cancer Inst Monogr 65: 391-396, 1984.

The title of this Symposium, "Use of Small Fish Species in Carcinogenicity Testing," presumably allocates a role to biochemical investigation, the degree of which depends on the questions for which answers are sought. Observation of neoplastic changes at the organismic and cellular levels after chemical insult would be the most direct and reliable indicator of carcinogenesis. Intermediate in scope is bacterial mutagenicity testing, which, in the presence of a causative agent, may show direct mutagenic activity. Should metabolic activation of this agent be necessary for activity, the test can be performed in the presence of an isolated MFO system of any chosen species. This would be followed by biochemical investigations of metabolic changes at the subcellular level. Because no link between biochemical lesions and cancerous growth can currently be made, biochemical indicators cannot stand by themselves. However, were one to predict occurrence of cancer by studying proliferative changes only, the complex biochemical–pharmacological interactions occurring before the onset of cancer would be ignored.

This point, then, represents the intersect between the disciplines of pathobiology and biochemistry and is the starting point for the search for biochemical indicators. Thus biochemistry serves to support the pathological findings until the biochemical target site of cell proliferation has been found. There are signs that this site has been located, but the full circle from cause to effect has not been completed. If one assumes for the sake of argument that this site were known, the complete metabolic fate of a cancer-causing agent will still have to be elucidated to estimate how much of it must be at the outside of the cell membrane to yield a certain amount at the target sites. Because alternative pathways for most biochemical reactions (activation or deactivation) exist, their influence will greatly affect the final concentration at the target site and their effects must be known. Biochemically speaking, most metabolic pathways are known; however, their quantitative and interactive aspects are not as well understood. Investigators performing biochemical studies often ignore the cell wall and membranous structures, assuming total availability within the cell. However, most "free" substances in a cell are not free at all but are bound to varying degrees and thus affect internal regulation. The knowledge of an ultimate target site and the molecular species involved would be much more useful if all events which lead to this state were understood. An understanding of this phenomenon would also open the door to pharmacological manipulations enhancing one pathway over another and possibly affecting the ultimate carcinogenic complex. It is within these realms that biochemical and pharmacological experimentation are needed.

To find biochemical indicators of the onset of cellular proliferation, one must show that the active form of the causative agent can be composed in the tissue in question. The primary pathway by which water-insoluble substances are altered metabolically is mediated by the MFO system located in the MSF of certain cells. This fraction of cell membranes is usually isolated by differential centrifugation under widely ranging conditions, such as pH, buffer type, centrifugation time, gradient material, ratio of tissue weight to homogenization volume, and the number of resuspensions of the final preparation (the last condition is of great importance because of the removal of soluble proteins). This procedure needs to be standardized. Following isolation, storage of the MFO should be at −80° C and not extend without activity check past 30 days; once thawed, it should not be refrozen. The final MFO preparation yields a membrane suspension, the protein concentration of which is established with difficulty. Bovine serum albumin, a soluble protein, is usually used as a standard in the

ABBREVIATIONS: MFO = mixed function oxidase; MSF = microsomal fraction(s); BP = benzo[a]pyrene; HPLC = high-performance liquid chromatography; 3-OH-, 5-OH-, or 9-OH-BP = hydroxy metabolites of BP; RFY = relative fluorescence yield; A = ampere(s); MCA = 3-methylcholanthrene.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported by the National Cancer Institute/Environmental Protection Agency Collaborative Program on Environmental Carcinogenesis, Project 3, Effects of Carcinogens, Mutagens, and Teratogens on Non-human Species (Aquatic Animals).

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[4] The author thanks Drs. D. R. Strength and S. Meera for their help.

measurement of insoluble membrane fragments, a practice which will not give accurate results. Inasmuch as we have few alternatives, a single method should be used to achieve comparable activities.

Additional complications arise during the final in vitro reaction of the MFO sample with a substrate. Wide variations occur in the uses of 1) concentration of co-factor (NADPH), 2) temperature and length of incubation, 3) introduction of water-insoluble test compounds into the reaction mixture, and 4) extraction and analysis of metabolites. All these can be standardized without much difficulty, and adjustments can be made when different animal species are used to yield optimal results. The analysis of metabolites, especially those of BP, has presented problems because the variety of analytical methods makes comparisons exceedingly difficult. Enzymatic activities have been based on "total extractable fluorescence" (*1*), disappearance of BP, and appearance of certain BP metabolites (*2*). The former type of analysis should be used only in a comparative sense because of its nonspecific nature and because fluorescence quenching and solvent effects are not defined. The measurement of the disappearance of the parent compound usually presents no great problem when reasonable care is taken, and results from different investigators can be compared. The most useful of all measurements without a doubt is the quantitative determination of all metabolites, particularly when other competing enzymatic reactions (conjugation) are done simultaneously with the oxidation. Total metabolite analysis is not as easy as it may appear, though cautious experimentation with an understanding of the underlying principles should give the desired results. Of greatest importance is that the analytical procedures be standardized so that the results of investigators can be compared.

Last but not least is the question of MFO induction in the laboratory situation. That MFO induction occurs in fishes and is caused by many of the same agents as in mammals is accepted by most working in this area of research. However, dosages and especially response times may differ among species. Administration of selected inducers is usually ip, im, or as an additive to food or water. The latter route would be the most preferable because it attempts to simulate field conditions, but facilities and initial trials favor the other methods. Any induction, either in the laboratory or field, should show changes in the metabolite pattern of a series of substrates. The work described here involves only 2 species of fish and only 1 carcinogen, BP; our intent was to find a fish species which could supplement the mammalian test species. The first choice was the mullet (*Mugil cephalus*) because it feeds by grazing on algae growing on rocks, skimming the surface of the water, and capturing any plankton of small size. When compared with the prey of a predator, this represents a much larger surface area of food to which highly adsorptive compounds are adsorbed. Mullet thus ingest toxicants such as BP adsorbed to the surface of its food. The major reason for our using the mullet as a test organism was the successful induction of the glucuronosyl and sulfate transferases (*3*). The MFO activity of the Gulf killifish is not as high as that of the mullet, and ip injection of an inducer is technically more difficult with the smaller fish. However, testing will continue with this and other small species.

MATERIALS AND METHODS

The 2 lobes of the mullet livers, dark red in color, represent from 1 to 2% of body weight, a convenient mass for the isolation of the MSF, the MFO activity of which has been described (*4*). Live capture and maintenance of mullet as test animals present difficulties. They are easily taken by gill netting, but removal from the net causes descaling and the animals usually die in a few days because of bacterial infection. The same problems exist with cast netting, even though our fish are collected in that fashion, and losses are high. Although we are able to keep survivors for up to 6 months during the cooler months, we know that they are nutritionally stressed (*4*). To induce the fish, we dissolved MCA and phenobarbital (acid form) in corn oil with the aid of sonification at 10-mg/ml concentrations to give solutions or suspensions to be administered to the fish at doses of 30 mg/inducer per kg of live weight. The first of 2 ip doses is given at day 0, the second on day 14, followed by termination at day 28. Fish were not fed but could obtain at least some food from the incoming seawater.

The MSF of livers were prepared by the method of Kinoshita et al. (*5*). The final MSF was resuspended in sucrose–Tris buffer and recentrifuged twice for removal of soluble proteins. Final preparations were in 0.05 *M* Tris, 0.25 *M* sucrose, pH 7.5, and stored at −80° C in small vials. Once thawed, contents were not refrozen. Determinations for MFO activity were made according to Selkirk et al. (*2*). Separation of reaction products was accomplished with HPLC. Identification and quantitation was based on the retention times and peak heights of mixtures of standard metabolites (National Cancer Institute) chromatographed under identical conditions. All solvents were of high purity suitable for liquid chromatography and were filtered through appropriate Millipore (0.25 μ) filters. Because the BP metabolite–DNA interaction is thought to present a critical step in the cause and effect of proliferative growth in mammalian systems, we performed preliminary physicochemical studies to see if such an interaction might also take place in fish. Highly polymerized salmon sperm DNA was dissolved in water at 0.1 mg/ml (sodium salt) and reacted with 9-OH-BP; then the RFY and changes in the spectrum of the 9-OH-BP were determined. The effects of methanol on the structure of the DNA and that of water on the fluorescence properties of the BP metabolite alone and in the DNA complex were carefully considered. We did another preliminary investigation in an attempt to establish whether complex formation with 9-OH-BP or an epoxide of BP would affect three-dimensional changes in the DNA. Inasmuch as we know that these changes can be followed with optical rotatory dispersion and circular dichroism, the spectra of the complexes were taken. The purity of all standard BP metabolites (National Cancer Institute repository) was checked by HPLC.

RESULTS AND DISCUSSION

In vitro activity determinations have been based mainly on the total metabolite evaluation or disappearance of BP,

TABLE 1.—*Partioning of 3-OH-BP from 1.0* N *sodium hydroxide*[a]

Phases	3-OH Species	Color	Polarity index[b]
Tetrahydrofuran	Φ–O⁻	Yellow	4.2
Aqueous sodium hydroxide	—	—	—
Hexane	—	—	0
Aqueous sodium hydroxide	Φ–O⁻	Yellow	—
Methyl ethyl ketone	Φ–O⁻	Yellow	4.5
Aqueous sodium hydroxide	<10% Φ–O⁻	—	—
Ethyl acetate	Φ–OH	—	4.2
Aqueous sodium hydroxide	—	—	—

[a] *See* text. Dashes = 0.
[b] Water = 9.

either of which can be misleading, and the results of the following experimentation must be taken into account when specific activities are resolved. The total metabolite evaluation is based on differential extraction with organic solvents from basic aqueous media. Fluorescence spectra of the phenols of BP differ greatly from those of their respective phenate anions, a property which one can use to follow extraction with certain solvents. With phenols, the pH of the reaction mixture as well as the solvent used for extraction must be carefully chosen for the analysis of the proper form of the compound. Table 1 shows a case in point when 3-OH-BP is partitioned from 1.0 *N* aqueous sodium hydroxide. The examples with tetrahydrofuran and ethyl acetate point clearly that, despite identical polarity indexes, the extracted forms of 3-OH-BP differ.

Were one to use fluorescence emission quantitation and a filter-type instrument, considerable error might occur (fig. 1). Further errors will occur if concentration-related quenching is not taken into account, e.g., 3,5- and 9-OH-BP isomers (table 2). Differences in the maxima of the RFY of these isomers are large, but they occur over practically the same concentration range. Another error can be introduced when methanol–water gradients are used during HPLC separations. Figure 2 shows the effect of water on the RFY of various phenols. The indicated values were obtained at concentrations that yield the maximum relative fluorescence, which, taken in methanol, served also as the 100% relative fluorescence points. Conditions for the diols and quinones have to be established.

Figure 3A shows the emission maxima wavelengths for the 12 isomeric phenol positions of BP. Positions 3 and 6 differ from the others, as they do when an RFY is assigned to the same positions (fig. 3B). Cautious interpretation supports free-radical action at the 6-position (*6*) and may explain high metabolite activity at the 3-position.

We made several attempts to gain insight into the DNA–metabolite interactions; the first was to use fluorescence to examine the physicochemical interaction that takes place. It was accomplished by our studying the change in the RFY. Table 3 shows that, at a weight ratio of approximately 1,000:1 of 9-OH:DNA under otherwise identical conditions, the RFY was much lower in the presence of DNA than in its absence. After 2 hours of UV irradiation, the RFY was reduced by about 5%. In the absence of DNA, under otherwise identical conditions, the RFY was reduced by more than 50% due to photolysis. Because the spectrum of the 9-OH was unchanged and little photolysis occurred, a charge-transfer type of interaction appears to have taken place. The second attempt focused on three-dimensional changes in the DNA molecule as a result of the complex formation shown above. The results revealed that no changes in the optical rotatory dispersion or in the circular dichroism spectra occurred at 25° C when salmon sperm DNA reacted with 9-OH-BP or the (*t*)-7,8-diol-9,10-epoxide of BP (fig. 4), which indicated no effect on the helix itself. Effects of higher temperatures are being studied.

In light of the above, metabolic studies were conducted

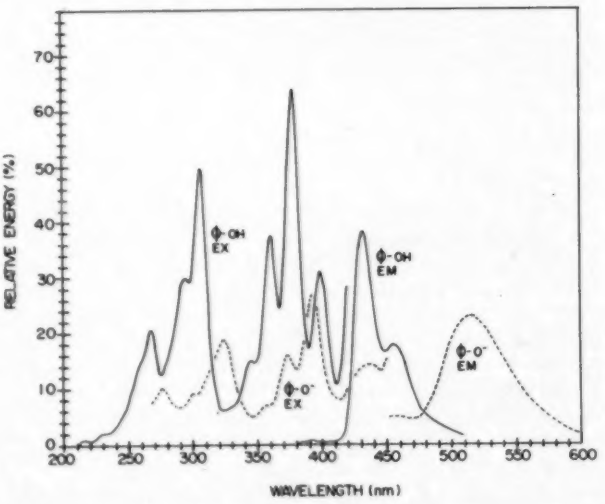

FIGURE 1.—Comparison of phenol and phenate spectra of 3-OH-BP. EX = excitation; EM = emission.

TABLE 2.—*Concentration-dependent fluorescence quenching in methanol*

BP metabolite	Concentration, ng/μl	RFY, μA/ng per μl
3-OH	0.666	11.3
	0.266	10.2
	0.133	12.9
	0.067	13.2
	0.033	13.0
	0.0067	9.0
5-OH	0.280	3.57
	0.112	4.10
	0.056	4.64
	0.042	4.52
	0.014	4.93
	0.0028	4.28
9-OH	17.0	11.1
	6.7	16.2
	0.40	14.8
	0.080	22.6
	0.040	22.0
	0.004	22.6
	0.002	20.5

with mullet induced with MCA. Earlier work (*4*) showed that induction required at least 14 days before MFO activity began to level off; later work (*3*) suggested that a second injection 14 days later and killing 14 days thereafter give more reproducible induction not only for the MFO activity but also for certain transferases. Following the analytical procedure of Elnenaey and Schoor (*7*), we determined MFO activity of MCA-induced mullet, sea catfish, and Gulf killifish. Using BP as substrate, we always found increased activity. Figure 5 illustrates the results of a 30-minute incubation with BP as substrate. Excellent separation was achieved with the newer instrument columns, with numerous metabolites shown, some of which await positive identification. The appearance of the 5-OH-BP among the metabolites of the MCA-induced mullet is unique and did not occur in the control animals, which

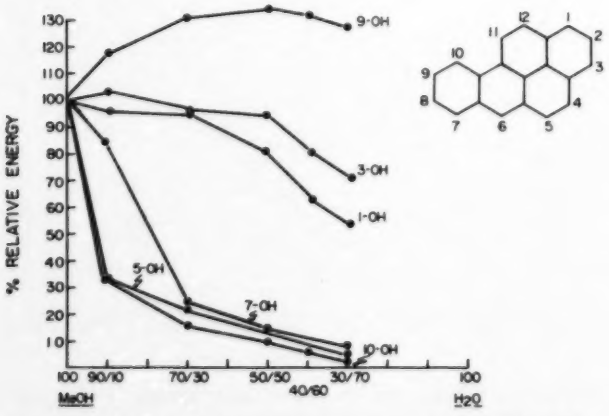

FIGURE 2.—Effects of water on fluorescence of some BP phenols.

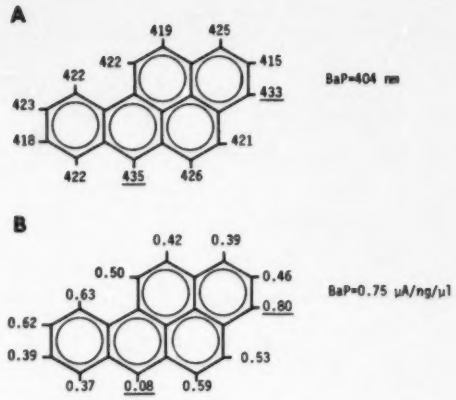

FIGURE 3.—A) Emission maxima (nm) in methanol of the isomeric phenol positions of BP (excitation at the highest wavelength, respectively). B) The RFY (μA/ng per μl) in methanol of the isomeric phenol positions of BP calculated from the emissions. BaP = BP.

indicated the possibility that at least for this species the appearance of 5-OH-type BP may signal induction by compounds like MCA. Of the quinones, only the 4,5-dione was positively identified when UV and fluorescence HPLC detectors were used. Compared with the other metabolites of BP, their UV extinction coefficients are high enough so that they can be seen easily by UV. They are highly unstable, especially in light, and must be purified before use as standards. Two groups of Gulf killifish were given ip injections of phenobarbital at rates of 12 and 25 mg/kg every 2 days for 12 days and then were killed after 14 days. Combined liver (5 fish) to body weight ratios of the fish were 2.2 and 1.4% for the 12- and 25-mg phenobarbital/kg doses and 1.0% for the controls. The MFO activities of the livers of the fish given the 12-mg/kg dose was significantly increased over those of the controls, although their activities were not as high as in the MCA-induced fish. No clear-cut induction was observed at the 25-mg/kg dose; perhaps toxic effects occurred at that level. Uridine-5′-diphosphoglucuronic acid-glucuronosyl transferase and sulfate transferase activities were also increased significantly over normal (*3*). When killifish were exposed

TABLE 3.—*RFY of 9-OH-BP in various mixtures of water, methanol, and salmon sperm DNA*[a]

Amounts of mixtures		RFY, μA/ng per μl
5 μl 9-OH-BP[b]	+ 195 μl methanol	13.2
5 μl 9-OH-BP[b]	+ 195 μl water	1.94
5 μl Methanol	+ 100 μl water 95 μl DNA (0.34 mg/ml)	0
5 μl 9-OH-BP[b]	+ 100 μl water 95 μl DNA (0.34 mg/ml)	0.26

[a] Measurement was made at 378 nm (422 nm emission).
[b] Amount of 9-OH-BP = 0.398 μg/μl.

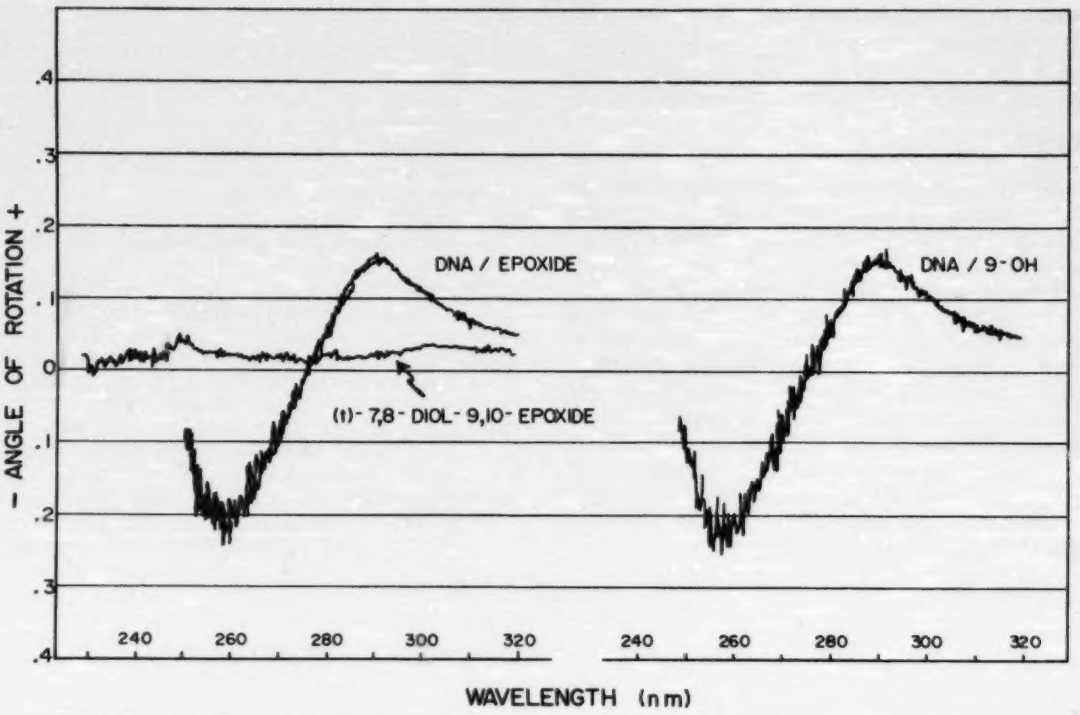

FIGURE 4.—Optical rotatory dispersion spectra of salmon sperm DNA reacted with A) 9-OH-BaP, and B) (*t*)-7,8-diol-9,10-epoxide of BP. BaP = BP.

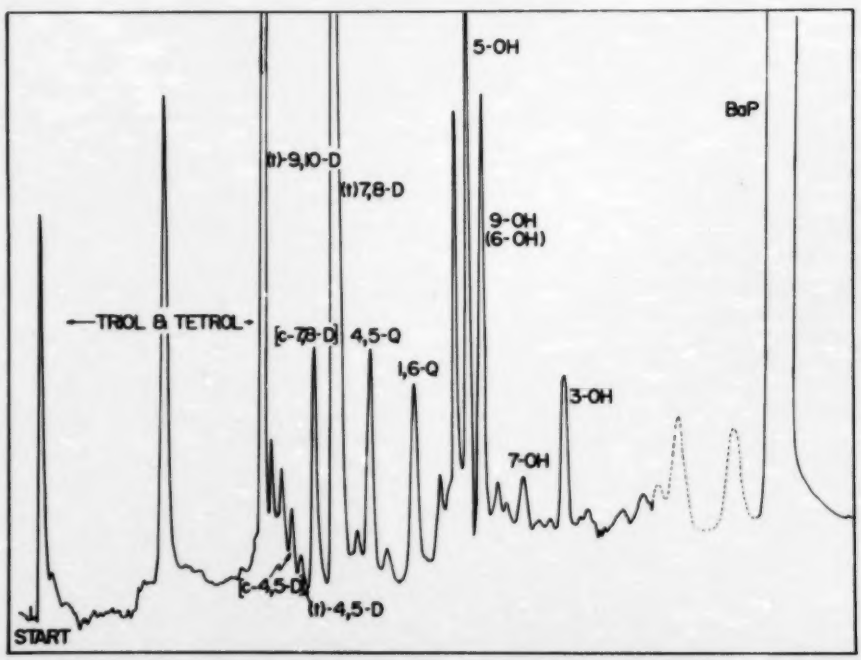

FIGURE 5. — Metabolites from MCA-induced mullet, 30 min MFO activity, with BP (BaP) as substrate.

to 0.3 and 0.03 mg phenobarbital/liter in flow-through exposures for 30 days, the percentages of liver to body weight of 8 fish averaged 2.1 and 1.9%, respectively, and MFO activities were slightly higher than those of controls.

SUMMARY

Few investigators doubt that fish are capable of metabolizing BP in much the same fashion as are mammals. Induction of MFO takes place, and metabolites are conjugated for excretion; care must be taken with the analytical biochemistry involved in their determination.

If species differences are to be discerned, individual metabolite patterns and conjugate formation must be established under various induction schemes. Therefore, investigators should standardize the analytical biochemistry as follows: induction as described here, incubation as in (*2*), MSF as in (*5*), and BP metabolite analysis as in (*2, 7*), and conjugation reactions according to Strength et al. (Strength DR, Daron HH, Schoor WP: Unpublished observations). This type of standardization would allow comparisons of their respective findings.

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